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Measure the Acute Toxicity of
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by

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FINAL REPORT:

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ABSTRACT

Research was undertaken to determine whether improvements were needed to increase the reliability of acute toxicity methodologies for mysid and juvenile Atlantic silverside waste characterization tests for municipal sewage sludge. Three new acute bioassays using mysids, larval fish and calanoid copepods were designed expressly to test sewage sludge and results using these tests were compared with results from tests presently used to regulate ocean dumping of sewage sludges. Similar results were obtained in an interlaboratory comparison of methods using the same sludge samples from three wastewater treatment plants. It is concluded that the present, easier to conduct methods may be satisfactory to characterize acute toxicity of sludge. Improvements in experimental design are recommended, however, to produce results suitable for statistical analysis.

The acute toxicity of sludge for the three treatment plants studied was low, with 96 h LC50 values of 1 to 10% whole sludge for fish, 0.5 to 2.5% for mysids and 0.03 to >0.8% for copepods. The copepods usually were more acutely sensitive to sludge than mysids by a factor of 6 to 9. The acute toxicity of sludge from a given treatment plant was found to be quite variable over time. One source of variation may be length of time the sludge is held in storage tanks. An order of magnitude increase in sludge toxicity was observed with storage of aerobically produced sludge at one plant. It is concluded that mysids and juvenile Atlantic silversides are appropriate test species to describe the relative toxicity of sludge, given the general suitability of these species for routine bioassays and their high to moderate sensitivity to chemical contaminants. However, the use of results of acute toxicity tests alone to evaluate environmental hazard is not considered appropriate.

1. INTRODUCTION

The Marine Protection, Research, and Sanctuaries Act of 1972 (MPRSA) (U.S. Congress, 1972) calls for the regulation of the transport and dumping of materials into ocean waters. The Act establishes a national policy to prevent or strictly limit the ocean dumping of any material which would adversely affect human health or the marine environment. The Act also establishes a permit program, to be administered by EPA, for ocean disposal of wastes which will not unreasonably degrade or endanger human health or the environment.

The procedures and criteria to be employed in issuing permits for ocean disposal are detailed in regulations published by U.S. EPA (1977). Bioassays are mandated to test the potential for significant undesirable effects of wastes, including the toxicity and bioaccumulation of contaminants. In practice, a waste is usually assessed through a two tier procedure. The first tier is waste characterization, to provide a preliminary description of physical, chemical, biological, and toxicological properties. Second tier studies are more detailed and are undertaken only if additional information is needed for a permit decision. These would include tests for chronic effects, bioaccumulation kinetics, and an assessment of the fate and transport of the waste. Operationally, these studies are most effectively conducted in a hazard assessment context. The central premise of hazard assessment is that information generated on the biological effects of the waste (effects assessment) will be interpreted in relation to a predicted or measured concentration of this waste in the environment (exposure assessment).

Ninety-six hour, acute bioassays typically are employed to describe toxicity of a material for first tier waste characterization. Acute

tests also may be required periodically during the life of a permit to monitor for changes in toxicity of the waste. The particular methods and species used in assays conducted for regulatory purposes are specified by the relevant permitting authority. At present, methods for such tests are developed largely from the guidance manual, "Bioassay Procedures for the Ocean Disposal Permit Program" (U.S. EPA, 1978). A limitation of the assays cited in this manual is that they were developed primarily for single compounds in the dissolved phase and not for particulate wastes containing a mixture of contaminants.

This project was undertaken to evaluate two aquatic animal bioassays presently required or accepted by regulatory authorities to measure acute toxicity of sewage sludge proposed for ocean disposal, the mysid and Atlantic silverside acute tests. Issues of concern include suitability of existing experimental designs, bioassay exposure systems, and test species to characterize toxicity of sewage sludge. Sludge may require special bioassay procedures, given its particulate nature, low toxicity, and high chemical oxygen demand (COD) and biological oxygen demand (BOD). A three phased approach was used for this evaluation. First, three new bioassays were developed at the EPA Environmental Research Laboratory, Narragansett (ERLN), expressly for sewage sludge. Next, response variability and sensitivity of these assays were examined by testing a number of sludge samples from one treatment plant. The third phase compared results of these refined bioassays with those of a contract laboratory, New York Testing Laboratories, Inc. (NYTL), using present U.S. EPA (1978) assay methods. Samples of the same sludge from three treatment plants were used in this study.

The ERLN bioassays incorporated many of the state-of-the-art procedures cited by the ASTM (1980) Standard Practice for Acute Assays. Test animals selected were larval fishes (Cyprinodon variegatus, Menidia menidia, and M. beryllina), mysid post-larvae (24 h) (Mysidopsis bahia) and copepods (Eurytemora herdmani and Pseudocalanus minutus). The static exposure method was adopted after it was determined that flow-through exposure systems could not reliably control the high concentrations of sludge required to obtain an LC50. Larval fish were selected because they may be more sensitive than the juvenile Atlantic silversides (M. menidia) presently used. Copepods were tested since they are ecologically highly significant in the ocean and they are more sensitive to some contaminants than presently used mysids (M. bahia). The copepod, P. minutus, which is common across the northeast U.S. continental shelf, was included to compare its acute sensitivity with that of the coastal copepod, E. herdmani. Microtox® (Beckman Instruments Inc., Carlsbad, CA) tests was also used in two studies.

2. METHODS

This section describes the refined ERLN 96 h static bioassays for sewage sludge using three groups of animals (larval fish, post-larval mysids, and copepods) and the 15 minute Microtox® test. Methods are described for collection, handling, and storage of sewage sludge; collection, holding and culture of bioassay animals; bioassay experimental design, preparation of sludge treatments and data analysis; and bioassay procedures. A table summarizing procedures used in the interlaboratory comparison are also cited and summarized in tabular form.

1. Sewage Sludge Collection, Handling and Storage

Sewage sludge used for assay development and the first set of definitive bioassays was obtained from the Middlesex County Utilities Authority, Sayerville, N.J. The manner of sample collection differed during the initial study period. Samples #1 through 7 were taken from the bottom of one of four storage tanks (samples #1 through 5 were used for preliminary studies only), sample #8 from the pipe carrying sludge to the storage tanks, #11 a composite from the bottom of two storage tanks, and #9B and 10 composites taken at the start, midway and end of pumping from two storage tanks to a barge. (Also see Appendix 1.) The latter procedure is used to sample barged sludge at this treatment plant for quarterly monitoring purposes. Sludge was transported to ERLN in polyethylene containers surrounded by ice and held in an insulated box. At the laboratory, sludge was poured through a 0.2 cm sieve to remove coarse material, stored in polyethylene containers and refrigerated at $4 \pm 2^{\circ}\text{C}$. Freezing did not occur.

Sludge samples used for interlaboratory comparison of methods were collected by treatment plant personnel. Each sample was a composite of three to five subsamples taken at regular intervals during pumping for barging. Each composite sample was split into two subsamples by NYTL, then handled as above by the two laboratories, except that subsamples were not sieved prior to use. The collection dates of all sludge samples tested are cited in Appendix 1.

2.2 Collection, Holding, and Culture of Animals

Fish

Adult Menidia menidia and M. beryllina were collected from the Pettaquamscutt River, RI, using a 30.5 m bag seine with 61 mm mesh. The fish were transported to the laboratory in 100 liter plastic barrels and ~30 held in the laboratory in 0.9 m diameter fiberglass tanks supplied with filtered seawater flowing at 1-3 liters min⁻¹. Water temperature was controlled at 15-17°C. The ambient salinity was 27-31 ‰. Holding tanks were illuminated by fluorescent lights (Cool White®, General Electric Co., Cleveland, OH) on a 12:12 L:D cycle. Fish were fed daily 15.5 g of Tetra SM80® (TetraWerke, Melle, Federal Republic of Germany) and 300-500 ml of concentrated 24 h post-hatch Artemia nauplii, Columbian strain, (cultured from ~3 to 5 ml of cysts).

Menidia spp. adults were held for spawning in 266 liter round fiberglass tanks connected to a closed-circulation seawater system as described by Middaugh and Takita (1983), or in tanks of the same size supplied with a continuous flow of filtered seawater of 1-3 liter min⁻¹. Seawater salinity varied from 28-32 ‰. Adults were brought into reproductive condition by changing the photoperiod to 14L:10D and changing water

temperature to $24 \pm 1^\circ\text{C}$. Fertilized eggs were deposited in "sock-sized" polyester fiber mats suspended at the surface of the water as in Middaugh and Takita (1983). Fiber mats with eggs were removed every two days, placed into static 4.5 liter hatching jars containing filtered seawater at $20 \pm 2^\circ\text{C}$. Larvae hatched within 8-12 days and were poured into static 56 liter glass aquaria containing clean filtered seawater. Newly hatched M. beryllina larvae were fed rotifers (Brachionus plicatilis) for the first five days post-hatch, then on days six and seven fed both rotifers and newly-hatched brine shrimp and after day seven fed newly-hatched brine shrimp alone. Newly hatched M. menidia larvae were fed only brine shrimp; rotifers were not required. All feeding was ad libidum. All static aquaria and hatching jars were aerated gently by air-stone.

Sheepshead minnow (Cyprinodon variegatus) larvae were obtained from laboratory spawned eggs, using culture methods similar to those of Hansen et al. (1978). Adult fish were collected by beach seine from a saltmarsh on Santa Rosa Island, Escambia County, FL, or from the Succotash Saltmarsh, Jerusalem, RI, and held in 160 liter aquaria supplied with flowing seawater (300 ml min^{-1}) controlled at $26 \pm 1^\circ\text{C}$ and 30 ± 2 ‰ salinity. Fish were contained in 2.5 mm mesh nylon baskets in the aquaria. Naturally spawned eggs fell through the mesh onto 250 mm collecting screens below the baskets. The screens were removed every two days and eggs washed into a shallow tray. Embryos were then pipetted into 19 cm glass finger bowls full of filtered seawater and incubated at 25°C , with gentle aeration. Water in the culture dishes was changed every two to three days. Larvae hatched in six or seven days and then were fed 24 h post-hatch Artemia daily.

Mysids

Mysidopsis bahia were cultured in flow-through 76 liter glass aquaria. The aquaria were filled with 60 liter filtered (15mm) natural seawater, which was continuously supplied at a rate resulting in 2-2.5 volume exchanges every 24 h. Standard aquarium undergravel filters provided aeration and a current conducive to feeding (Gentile et al., 1983). Undergravel filters were covered by a coarse (2-5 mm) dolomite substrate one inch deep, prewashed in deionized water. Cultures were maintained at $25 \pm 1^{\circ}\text{C}$, 30 ± 2 ‰ salinity, with fluorescent lights (Cool White®) on a of 14L:10D photoperiod with dawn and dusk simulation.

Twenty-four hour post-hatch nauplii of Artemia salina (Brazil strain) were added as food organisms using an intermittent flow feeder which provided approximately 7×10^4 nauplii day^{-1} for each 76 liter culture (Johns et al., 1981). This ration was adjusted according to mysid density, so cultures were always fed ad libitum in order to limit cannibalism of young. Mysid cultures were cropped at least monthly to maintain an optimum density of c.a. 20 mysids per liter. At this density, at least 70% of the females have eggs in the brood sac; at higher culture densities reproduction is depressed. Hydroids or worms growing in the culture tanks were removed by scraping the sides of the tanks weekly and siphoning.

Mysid post-larvae (24 h post-hatch) were collected for bioassays by holding gravid females overnight in separate netted (1000mm) flow-thru containers held within a 4 liter separatory funnel (Fig. 1). Ad libitum was maintained. Newly hatched post-larvae passed out of the netting into the funnel. Mysid post-larvae were removed by opening the stopcock, collected in another container, then transferred to bioassay exposure cups using a wide bore pipette (≥ 4 mm i.d.).

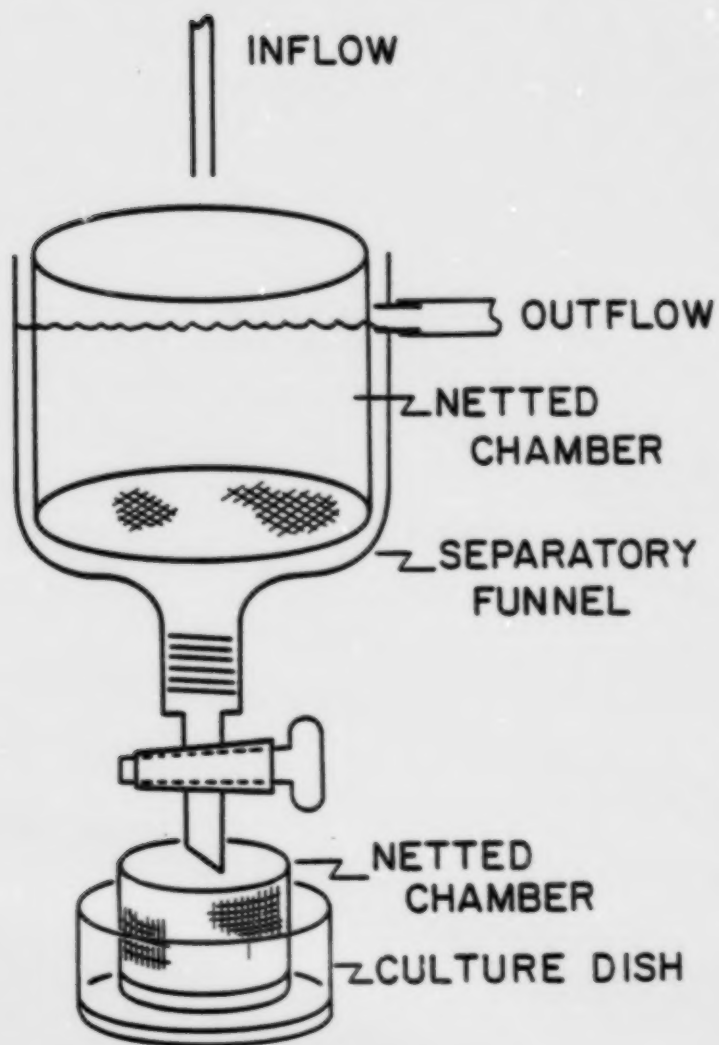


Figure 1. Apparatus for collection of post-larval mysids from gravid females.

Copepods

Eurytemora herdmani were obtained from a breeding laboratory population established at ERLN in June, 1982, from young of approximately 100 females collected from Narragansett Bay, RI. Copepods were cultured statically in 0.45 mm filtered 15°C, 25-30 ‰ salinity, using Pettaquamscutt River, RI, water. Copepods were reared in groups, initially comprised of nauplii hatched within a seven day period. Copepods were held in 11.4 cm Carolina® culture dishes (Carolina Biological Supply Co., Burlington, NC) containing approximately 300 nauplii, 200-250 copepodids, or 150 adults per dish. The copepod culture medium was changed weekly.

All copepod life stages in the laboratory culture were fed once a day, six days per week, with a mixed algal diet of Rhodomonas baltica, Skeletonema costatum, and Pavlova lutheri combined in approximately equal amounts by volume. Algal food density varied with life stage: nauplii, 0.25 ml algal mix 100 ml⁻¹ seawater; copepodids, 0.75 ml 100 ml⁻¹ seawater; and adults, 0.55 ml 100 ml⁻¹ seawater. Phytoplankton were cultured in 100 ml lots of a modified f/2 medium (Guillard and Ryther, 1962). Algal medium modifications were made to the trace metal mix; only the prescribed EDTA, iron, zinc, and manganese were added. No other buffers or chelators were included. Silicates were added at half strength. Algae were grown at 20°C under 24 h fluorescent illumination (Cool White Sho®, Westinghouse Electric Corp., Pittsburg, PA) for the first seven days and then were placed at 15°C, 12L:12D fluorescent illumination. Only phytoplankton cultures between seven and fourteen days old were fed to copepods.

Female Pseudocalanus minutus used in the bioassays were collected from lower Narragansett Bay during the winter 72 to 96 h prior to the

test. In the laboratory, animals were held at 10° in filtered, 30 ‰ Pettaquamscutt River water in lots of 50 animals per 150 ml water. Copepods were fed daily on a mixed diatom diet of Skeletonema costatum, Thalassiosira pseudonana, and Thalassiosira weissflogii.

2.3 Experimental Design, Preparation of Sludge Treatments, and Data Analysis

Experimental design for the ERLN bioassays consisted of one control treatment and at least five sludge concentrations. At a minimum, duplicate exposure chambers were used for each concentration, with a total of at least 20 animals per concentration. Organisms were distributed randomly into exposure chambers which were, except for the copepod assays, randomly placed on exposure tables. Specifics of each bioassay design are given in Table 1.

Sewage sludge treatment concentrations were selected on the basis of prior experience and modified as necessary when initial bioassays on a given sludge sample were completed. Range-find assays were usually not conducted. A wide range of concentrations were used due to the relatively low toxicity, yet variable nature of sewage sludge. The following ranges usually spanned the LC₅₀ for whole sludge: fish, 0.5 - 10%; mysids, 0.1 - 4%; copepods, 0.01 - 0.4%. The particular test concentrations used were determined by logarithmic series (Rand et al., 1975). Sludge treatments were prepared on a volume to volume basis with a specified volume of well mixed sewage sludge diluted in a known volume of sand filtered (15 mm) Narragansett Bay water (25-30 ‰ salinity). All treatments were aerated for 30 min (for mysids) or at least 60 min (for copepods and fish) before the animals were added. New treatment

Table 1. Summary of Acute Test Methods for Sewage Sludge Employed by New York Testing Laboratory (NYTL) and Environmental Research Laboratory-Narragansett (ERLN). Exceptions noted in text.

	<u>Fish Bioassay</u>	
	<u>NYTL</u>	<u>ERLN</u>
Species	<u>Menidia menidia</u>	<u>Menidia menidia</u> <u>M. beryllina</u> <u>Cyprinodon variegatus</u>
Life-History Stage	Juvenile	<u>Menidia</u> : 2-4 wk. larvae <u>Cyprinodon</u> : newly hatched
Source	Field collected	Laboratory cultured
Pre-Exposure Holding	≥ 14 day	1 to 24h
Exposure Mode	Static, no renewal	Static, no renewal
Exposure Chamber	Aquarium, 38 liter	Crystallizing dish, 1.2 liter
Experimental Design:		
No. treatments inc. control	4 to 6	6 to 7
No. organisms per chamber	10	12
No. treatment replicates	1	2
Test Solution Vol.	15 liter	1 liter
Treatment Aeration:		
Pretest	None	> 1 h
Test	None and 100 bubbles min^{-1} by pipette	Vigorously by pipette, D.O. $> 85\%$ saturation
Seawater	Natural, 5 μm diatomaceous earth filtered	Natural, 15 μm sand filtered
Temperature	Ambient ($\sim 18-20^\circ$)	$22 \pm 2^\circ\text{C}$
Salinity	27 ± 2 ‰	31 ± 4 ‰
Illumination	Ambient	100 LX Cool White, 12L:12D
Test Food	None	<u>Artemia</u> nauplii daily

(Sheet 1 of 3)

Table 1. (Continued)

Species	<u>Mysid Bioassay</u>	
	<u>NYTL</u>	<u>ERLN</u>
	<u>Mysidopsis bahia</u>	<u>Mysidopsis bahia</u>
Life-History Stage	1-2 day post-hatch	1 day post-hatch
Source	Laboratory cultured	Laboratory cultured
Pre-Exposure Holding	24 h	None
Exposure Mode	Static, no renewal	Static, no renewal
Exposure Chamber	Crystallizing dish, 2.6 liter	Crystallizing dish, 2.6 liter, containing 3 sub-chambers
Experimental Design:		
No. treatments inc. control	4 to 6	6
No. organisms per chamber	15	15 (5 per sub-chamber)
No. treatment replicates	2	2
Test Solution Vol.	1 liter	1 liter
Treatment Aeration:		
Pretest	None	30 min
Test	100 bubbles min ⁻¹ by pipette	Air lift
Seawater	Natural, 5 µm diatomaceous earth filtered	Natural, 15 µm sand filtered
Temperature	Ambient (~18-20°)	25 ± 1°C
Salinity	27 ± 2 ‰	30 ± 2 ‰
Illumination	Cool White, 14L:10D	100 LX Cool White, 14L:10D
Test Food	<u>Artemia</u> nauplii daily	<u>Artemia</u> nauplii daily

(Sheet 2 of 3)

Table 1. (Concluded)

<u>Copepod Bioassay</u>	
<u>ERLN</u>	
Species	<u>Eurytemora herdmani</u> <u>Pseudocalanus minutus</u>
Life-History Stage	Adult, female
Source	<u>E. herdmani</u> : Laboratory cultured <u>P. minutus</u> : Field collected
Pre-Exposure Holding	<u>E. herdmani</u> : 1-5 day post-maturity <u>P. minutus</u> : 3-4 day post-maturity
Exposure Mode	Static, 24h renewal
Exposure Chamber	Erl. flask, 250 ml
Experimental Design:	
No. treatments inc. control	7
No. organisms per chamber	5
No. treatment replicates	4
Test Solution Vol.	100 ml
Treatment Aeration:	
Pretest	60 min
Test	Orbital shaker
Seawater	Natural, 15 μ m sand filtered
Temperature	12 $\pm$ 1°C
Salinity	28 $\pm$ 2 ‰
Illumination	Cool White Sho, 12L:12D
Test Food	None

(Sheet 3 of 3)

solutions were prepared daily for tests having 24 h waste renewal. Exposure concentrations in the copepod assays were measured gravimetrically at least once during each test. (See Appendix 2 for procedures.)

Results are presented as LC50 values. These values were estimated using a program which computes the LC50 by the probit (Finney, 1971) and moving average (Thompson, 1974) methods if the condition of two partial kills is met, and by the binomial test method (C.E. Stephan et al., unpublished). The program includes Abbott's formula to adjust for control mortality. Missing animals were counted as dead when analyzing results of the larval fish and mysid tests; missing copepods were subtracted from the initial sample size before data analysis. No LC50 values are reported for larval fish or mysid tests with control mortality exceeding 10%. A copepod test with control mortality below 10% was not achieved for some sludge samples, as noted in the results.

2.4 Test Procedures

Test conditions and procedures for the ERLN and NYTL 96 h tests are cited in Table 1. The ERLN tests were conducted largely according to ASTM Standard Methods (1980). Additional details of test equipment, procedures for animal and waste handling, test monitoring, and animal enumeration are given in Appendix 3. Three exceptions to procedures cited in Table 1 occurred in copepod tests on sludge samples #6 through 11. In these tests, whole sludge was aerated vigorously for at least 30 minutes before, not after dilutions were mixed, and copepods were added within 30 min of preparing treatment solutions. Also, the dilution water was collected the day before and equilibrated to the test temperature (12°).

Sludge was prepared for the Microtox® test by centrifuging at 2000 X G (5000 RPM) for 5 min at 5°C. The supernatant was tested for toxicity by standard Microtox technique as described in the Beckman Microtox® System Operating Manual (Beckman Instruments Inc., 1982). The exposure duration was 15 min. The salinity was adjusted with Microtox® osmotic adjusting solution. Absorbance correction was generally not required.

2.5 Interlaboratory Comparison for Test Methods

Bioassays using the refined ERLN methods were conducted during the same time period (+ 2 weeks) that NYTL performed quarterly sludge bioassays on sludge from three treatment plants. Subsamples of test sludges were obtained from NYTL for the Narragansett bioassays. NYTL methods were developed from the U.S. EPA (1978) bioassay guidance manual and are described in the NYTL Manual of Practice (unpublished). (Appendix 4.)

3. RESULTS

Evaluation of ERLN Bioassay Methodologies for Sludge

A limited series of static, acute 96h bioassays were conducted between April, 1983 to February, 1984 to evaluate the need for improvements in the ERLN methods to measure sewage sludge toxicity. These assays examined test replicability, the need for 24h renewal vs. nonrenewal of sludge in 96h assays, relative sensitivity of the test species to sludge and variability in toxicity of different sludge samples from the same plant.

Results of 96h bioassays (Table 2) suggest good within-sample replication for mysid and larval fish tests. For example, the ratio of the highest to lowest LC50 values for replicate tests on a given sludge were 1.5 for Cyprinodon variegatus, 1.7 for Menidia menidia, and 1.1 and 1.6 for Mysidopsis bahia.

Results from 96h static toxicity tests using M. bahia both with and without 24h renewal of sludge solutions (Table 2) suggests that renewal is not required to insure maintenance of contaminant concentrations for this sludge. LC50 values from triplicated 96 h tests receiving renewal ranged from 2.4 to 2.6 vs. 2.6% whole sludge for a test without waste renewal. The concentration response curves (Fig. 2) for these assays also demonstrate the good replication between renewed and nonrenewed static tests on this particular sludge sample.

There is considerable variation in LC50 values for tests on different sludge samples from the same treatment plant (Table 2 and 3), in contrast to good agreement of replicate tests on the same sample. LC50 values for two samples tested with mysids differed by a factor of 5 (LC50 range: 0.51 - 2.6%), while copepod results for four different sludge

Table 2. Results of Initial Definitive Static Acute Bioassays Using Refined ERLN Methods for Sewage Sludge. LC50 as % Whole Sludge (95% C.I.) Calculated by Probit (P), Binomial (B), or Moving Average (M) Method After Abbott's Correction for Control Mortality. All Sludge Samples From Middlesex County Utilities Authority.

Sample #	Sample Type	Larval Fish			Mysid	Copepods	
		<u>C. variegatus</u>	<u>M. menidia</u>	<u>M. bahia</u>	<u>E. herdmani</u>	<u>P. minutus</u>	
6	Storage Tank	-	-	-	-	0.19(0.12, 1.0)B	
7	Storage Tank	-	-	2.5(1.0, 8.6)Pt 2.6(1.9, 3.3)Mt 2.3(0.02, 6.8)Pt 2.6(1.9, 3.5)P	0.19(0.13, 0.29)P	-	
8	Line to Storage Tanks	5.0(4.10, 6.1)P 7.5(5.60, 10.0)P	3.9(1.8, 5.6)B 6.5(5.6, 10.0)B	-	-	-	
9B	Barge Compo-site	-	-	-	< 0.08 < 0.02 < 0.02	-	
10	Barge Compo-site	-	-	-	0.03(0.01, 0.45)P 0.09(0.05, 0.15)P*	-	
11	Storage Tank Compo-site	> 10.0	-	0.84(0.49, 1.1)P 0.51(0.35, 0.67)P	0.36(0.02, 0.50)P 0.10(0.08, 0.14)P*	0.11(0.08, 0.15)P 0.13(0.1, 0.18)P*	

* control mortality > 10%

† sludge treatments renewed each 24h

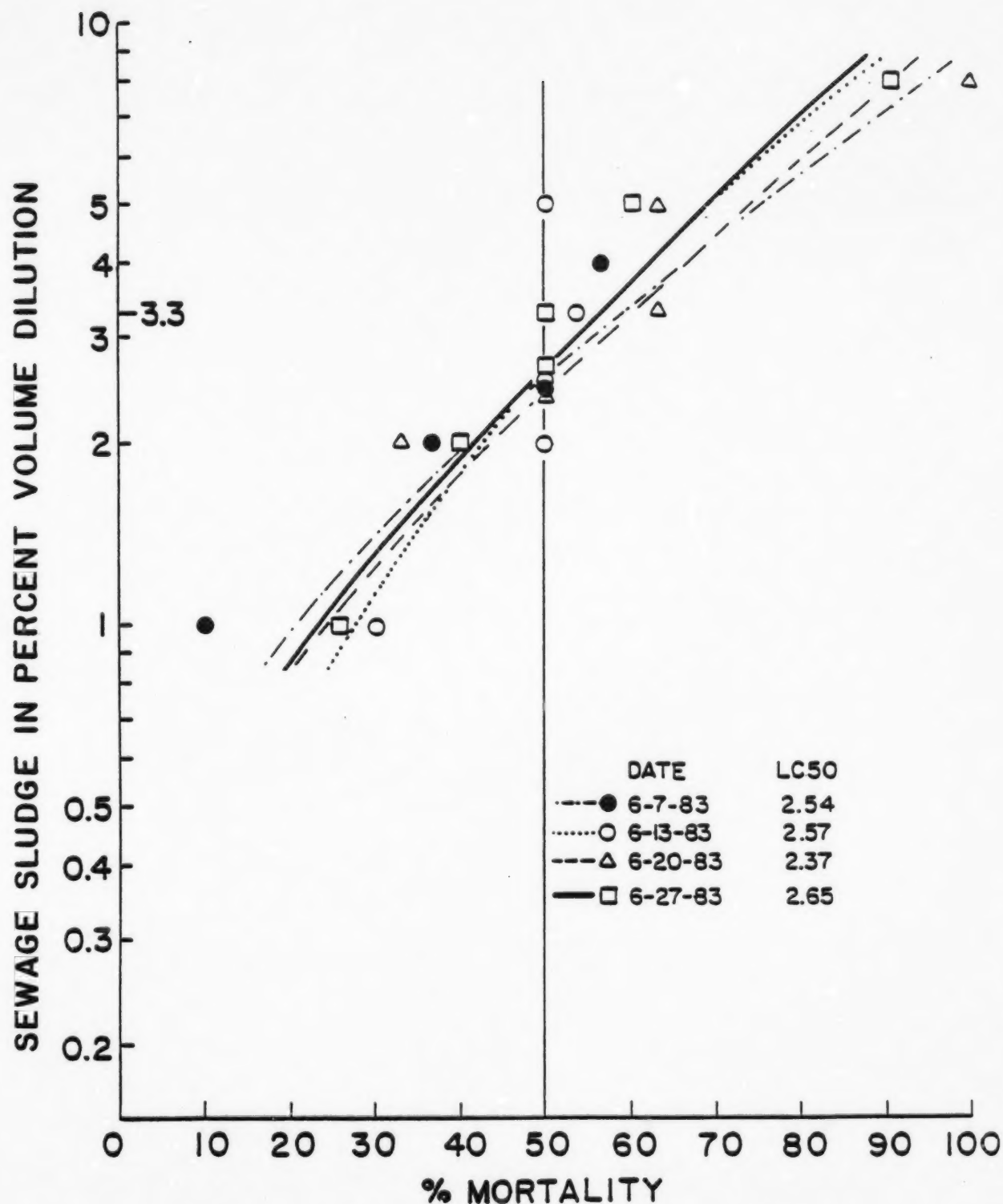


Figure 2. Concentration response curves of sequential static acute toxicity tests on sewage sludge (sample #8) using *Mysidopsis bahia*. All tests with 24 h renewal of the waste, except 6/27/83.

Table 3. Toxicity of Middlesex County Sewage Sludge Collected at Different Locations on Two Successive Days. Microtox EC50 = % of Sludge Sample Causing 50% Inhibition of Bacterial Fluorescence. July 18 Tank 2 and 4 Samples Collected While Pumping Sludge From Tanks For Barging.

[illegible]

samples had a range exceeding one order of magnitude ($< 0.02 - 0.36\%$). This variability may be in part due to different sampling procedures and locations used during this initial study. A subsequent Microtox® toxicity survey suggests that the toxicity of sludge may increase appreciably in storage tanks (Table 3). During this survey, sludge entering the storage tanks from the gravity thickener had an EC50 value of approximately 30% whole sludge, while the toxicity increased as much as an order of magnitude in the storage tanks, the EC50 values ranging from 2.1 - 9.1%. It also appears that toxicity of sludge increases with storage time, since three of four sludge samples showed lower EC50 values when the same section of the storage tank was sampled 24 h later. The 95% confidence intervals for this Microtox® test were calculated to be $\sim \pm 10\%$ of the reported values.

Sludges tested during the initial toxicity study differed in the time, location and method of sampling. As illustrated by the storage tank toxicity study, these sludges may have differed greatly in toxicity, limiting any comparison of these results to within-sample comparisons only. The species sensitivity indicated by tests on sample #11 (Table 2) suggest the following relationship: copepods > mysids > fish larvae. Also, tests on samples #8 and 11 indicate no appreciable differences in sensitivity between the two species of fish or two species of copepods tested (Table 2).

Comparison of Refined and Presently Used Sludge Bioassays

The 96h LC50 values obtained by the refined ERLN methodologies were slightly higher for each sludge sample with mysids and for two of three samples tested with fish when compared with results of the technically

easier contract laboratory test methodologies (Table 4). These differences are statistically significant for fish and mysid tests on Bergen County sludge and the fish test with Middlesex County sludge, as indicated by nonoverlapping 95% confidence intervals. Nonetheless, the mysid and fish test results are considered comparable between the two laboratories, with one exception. Importantly, except for the fish test with Middlesex sludge, these interlaboratory results are within a factor of 2.4 or less. An EPA interlaboratory comparison study using similar 96 h static acute test protocols found that LC50 values determined by six different laboratories varied by a factor of 2.2 for mysids and 2.5 for the fish Cyprinodon variegatus for an inorganic compound and by at least a factor of 2.9 for an organic chemical (Schimmel, 1981). In contrast, the different LC50 values obtained by the two fish assays on the Middlesex County sample may well be biologically significant. However, it cannot be determined from this study whether this difference is due to differences in bioassay protocols per se or due to other variables (e.g., source of dilution water, source of test organisms, condition of test organisms, different test species, etc.).

Copepods were more sensitive than mysids tested at ERLN by a factor of 5.7 for sample #13 and 9.3 for sample #14. This is consistent with the greater sensitivity observed with copepods (by a factor of 5 to 14) during initial definitive bioassays on Middlesex County sludge (Table 2). Relative sensitivity of copepods and mysids to Westchester County sludge cannot be determined. Here, the mysid LC50 value was 1.8%, while only 35% of the copepods in 0.8% sludge died. Control mortalities in copepod assays were: 13%, sample #13; 20%, sample #14; 15%, sample #15.

Table 4. Results of Split-Sample Study Comparing ERLN Refined Sewage Sludge Acute Bioassay Methods with the New York Testing Laboratory (NYTL) Methods and Microtox. LC50s as % Whole Sludge, Calculated by Probit (P), Moving Average (M) or Binomial (B) Method after Abbott's Correction for Control Mortality; 95% C.I. in ().

Sludge Sample #	Treat-ment Plant	Fish		Mysids		Copepods	
		Juvenile <u>M. menidia</u>		<u>Mysidopsis bahia</u>		<u>E. herdmanni</u>	
		NYTL	ERLN	NYTL	ERLN	ERLN	Microtox (EC50)
13	Bergen Co.	2.1(1.6,2.7)P	5.0(3.3,6.7)P	0.91(0.70,1.4)P	1.9(1.5,3.1)P	0.34(0.28,0.47)H*	4.5
14	Middlesex Co.	1.0(0.78,1.3)P	8.1(7.5,8.8)P	0.66(0.49,1.3)P	1.1(0.76,1.7)P	0.12(0.10,0.15)P*	4.5
15	Westchester Co.	2.6(1.5,5.0)B	2.3(1.7,3.1)B	0.85(0.51,1.2)P	1.5(0.28,3.6)P	35% mortality at 0.8%*	4.5

* control mortality 13 to 20%

The Microtox® EC50 was 4.5% for each of the three samples tested in the interlaboratory study. The uniformity of this value between these samples corresponds with good between-sample agreement of mysid assays conducted by NYTL.

An Analysis of Historical Data

Additional acute toxicity data determined by NYTL for sewage sludge from seven metropolitan NY and NJ treatment plants are summarized in Table 5 in order to evaluate results of our study in the context of a more extensive data base. Data for this table were compiled from quarterly toxicity results using present regulatory tests on composite samples of barged sludge. Close agreement is evident between NYTL LC50 values from our comparative study for samples #13, 14 and 15 (Table 4) and mean values in Table 5 for the Bergen County, Middlesex County and Yonkers (Westchester County) treatment plants. This suggests that the acute toxicity of sludge samples used in the interlaboratory comparison study was typical for these three plants. Yet Table 5 also shows the acute toxicity of sludge from a given treatment plant may vary greatly over time, as indicated by high within-plant coefficients of variation. Greater acute sensitivity of mysids to sludge seen here relative to Atlantic silversides is consistent with results of the present study.

Table 5. Acute Toxicity of Sewage Sludge Reported by New York Testing Laboratory for Seven Metropolitan NY and NJ Wastewater Treatment Plants. LC50 Values Calculated by Binomial Method.

Treatment Plant	Mean LC50 $\pm$ S.D.(N) Coeff. of Variation		Ratio LC50 Silversides/ Mysids for same sample $\frac{\bar{x}}{s}$ (range)	N of Sample Mysid LC50 < Silverside LC50
	Mysids	Atlantic Silversides		
Middlesex Co. 3/82 - 4/84	0.42 $\pm$ 0.23(8) 55	1.3 $\pm$ 0.38(7) 29	3.2(1.5-6.2)	100
Newtown Creek* 6/80 - 10/82	0.70 $\pm$ 0.53(15) 76	1.3 $\pm$ 0.33(14) 25	3.5(0.5-14.4)	86
Bergen Co. 3/81 - 3/84	0.72 $\pm$ 0.22(12) 31	2.2 $\pm$ 1.3(11) 56	3.5(1.7-7.8)	100
Westchester Co. 1/81 - 4/84	1.0 $\pm$ 0.5(12) 50	2.2 $\pm$ 0.83(10) 38	2.1(0.6-3.5)	89
Hunts Point* 6/80 - 10/82	1.1 $\pm$ 0.48(14) 42	1.6 $\pm$ 0.71(14) 44	1.4(0.6-3.0)	79
Wards Island* 1/81 - 10/82	1.2 $\pm$ 0.27(13) 22	2.0 $\pm$ 0.96(12) 48	1.8(0.7-2.8)	72
Rockaway* 1/81 - 10/82	1.6 $\pm$ 0.84(13) 54	2.0 $\pm$ 0.58(12) 29	1.4(0.5-2.6)	70

*Data cited in City of New York (1983).

4. DISCUSSION

The initial series of bioassays on Middlesex County sewage sludge conducted during development of the refined ERLN methods provides some insight into the performance of these assays. Where data are available, there was good within-sample replication in ERLN mysid and larval fish assays. Copepod assays were more variable, as two out of three varied by a factor of 3. Acute toxicity of Middlesex County sludge was low for fish and mysids, with 3.9% whole sludge the lowest LC50 value for fish and 0.51% for mysids. However, copepods were more sensitive; LC50 values ranged from $< 0.02 - 0.36\%$.

The comparison of ERLN refined and presently used regulatory sewage sludge assays indicates that these two methods produce comparable results, with one exception, but improvements in the experimental design of the regulatory assay are needed. Although sludges from only three treatment plants were included in the interlaboratory study, the absence of major differences indicate the fish and mysid assays presently used for regulation are satisfactory for waste characterization. However, a review of the quarterly sludge bioassay reports submitted to EPA revealed minor improvements in experimental design should be implemented to insure results are reliable. U.S. EPA (1978) or other accepted test guidelines were not followed consistently. Therefore, the experimental design was insufficient to produce concentration response data which could be tested statistically. As a consequence, this absence of statistical confidence invalidates comparisons between the toxicity of different samples from the same plant, samples from different plants and the sensitivity of test species. We recommend that the number of waste concentrations and their dilutions be selected so they are sufficient to produce partial kills

of tested species to adequately bracket the LC50 and that the number of individuals per treatment be increased. To produce at least two partial kills, standard methodologies (e.g., ASTM, 1980) recommend a minimum of five waste concentrations spaced at dilutions of 0.6 of the highest concentration, plus a control. We found dilutions of 0.5 are sufficient with sludge. In some tests, the contract laboratory used only three waste treatments and some dilutions were as great as a factor of 0.3. The precision of acute tests on sewage sludge may also be improved by using more organisms. Further discussion of these issues is provided in Appendix 5.

One application of acute toxicity data from sewage sludge tests is to regulate the rate of sewage sludge discharge from barges during ocean disposal. The ocean dumping regulations (U.S. EPA, 1977) establish a Limiting Permissible Concentration (LPC) of a waste which cannot be exceeded either outside of the dump site during dumping or within the dump site after an initial 4 h mixing period. EPA Region 2 establishes an LPC for sewage sludge based on acute bioassays with the whole waste. The LPC is calculated as 0.01 of the second lowest LC50 reported by each discharging treatment plant during the previous two years. Atlantic silverside or mysids are normally more sensitive to sewage sludges than other commonly used tests (e.g., the phytoplankton, (Skeletonema costatum) or grass shrimp (Palaemonetes pugio) test). Hence, these LC50 values are typically used to calculate the LPC for various metropolitan NY and NJ sludges. The following questions are relevant to evaluate these assays for this application: (1) are the test species "appropriate sensitive marine organisms" as defined by the regulations; (2) is the

procedure for obtaining the LPC scientifically defensible; and (3) does it provide protection from unreasonable acute and chronic toxicity in tested and untested species of ecological or economic value? The latter question is the most difficult to answer.

Ocean dumping regulations call for use of bioassay species which are among the most sensitive as documented in the scientific literature or species accepted by EPA as being reliable to determine the anticipated impact on the ecosystem at the disposal site. Few acute toxicity data are available for the same sewage sludge sample to compare sensitivity of species representative of dump sites with the sensitivity of mysids and Atlantic silversides. The present study showed copepods may be more sensitive to sewage sludge than mysids by a factor of 6 to 9 and mysids more sensitive than larval fish typically by a factor of 2. Quarterly sludge monitoring data obtained for EPA Region 2 (Table 5) show mysids were more sensitive than juvenile Atlantic silversides in 75% of 52 tests of sludge from four City of New York wastewater treatment plants. At the other three treatment plants cited here, mysids were more sensitive in 95% of the tests (26 out of 27). Mysid LC50 averages were lower than Atlantic silversides by a factor of 1.6 to 4 for the seven treatment plants. A study by Ecological Analysis, Inc., of sludges from 12 City of New York treatment plants showed mysids to be most sensitive in 91.5% of 35 tests on whole sludge, Atlantic silversides most sensitive in 8.5%, and adult grass shrimp (Palaemonetes pugio) always least sensitive (Fava et al., in press; City of New York, 1983). These mysid bioassays differed from those conducted by ERLN and NYTL in that no supplemental aeration was provided, therefore, results may not be directly comparable. Franklin

(1983) found larval brown shrimp (Crangon crangon) were up to 500 times more sensitive to sewage sludge than adults after 24 h exposure. In a series of 96 h exposures to 10 sludges from the United Kingdom, Franklin found adult brown shrimp most sensitive of five species tested, with the sensitivity relationship being: brown shrimp > armed bullhead > cockle > mussel and periwinkle snail.

Although available information is quite limited, at present it appears reasonable to consider the mysid (M. bahia) and Atlantic silverside (M. menidia) as appropriate marine organisms for acute regulatory tests for waste characterization of sewage sludge. They are used in marine testing because they are available through culture or capture, survival is acceptable in culture and testing, and they are reasonably sensitive. These species are not as sensitive as copepods nor possibly developmental stages of certain crustaceans. However, a review of acute toxicity tests cited in selected EPA water quality criteria documents showed mysids were on the average more sensitive than 73% of the species tested against metals and were more sensitive than 54% of the species tested against pesticides (Hansen, in press). Also, Anderson et al. (1974) found a congeneric species, M. almyra, to be most sensitive of six marine species to acute exposures to a number of oil-in-water dispersions and water-soluble fractions of four different oils. Regarding the relevance of these species to ocean dump sites, the Atlantic silverside inhabits the northeast U.S. continental shelf out to 50 m during the winter, but in other seasons inhabits intertidal creeks, marshes and the shore zone of estuaries (Conover and Murawski, 1982). The mysid, M. bahia, only occurs in shallow tropical and semi-tropical bays (Stuck et al., 1979). However, M. bahia

may be a reasonable surrogate for U.S. continental shelf mysid species for toxicological purposes. Acute and chronic bioassays with cadmium produced comparable results with M. bahia and M. bigelowi (Gentile et al., 1982). The latter species ranges along the entire eastern U.S. coast and on Georges Bank.

The extent that the LPC may be protective of adverse acute and chronic effects on tested or untested species at an ocean disposal site is largely untested. The 0.01 factor used to calculate the LPC from acute toxicity tests was not developed experimentally to account for variability in acute sensitivities of untested species, nor to account for differences between concentrations that are acutely lethal and concentrations likely to protect species from ecologically important sublethal effects, including bioaccumulation of contaminants associated with sludges. The 0.01 factor cited by the ocean dumping regulations was adopted from FWPCA (1968) Water Quality Criteria. This generalized factor was recommended to estimate chronically acceptable concentrations of an individual persistent chemical or waste when only acute toxicity was known. The utility of this factor to estimate chronically acceptable concentrations of sewage sludge has not been determined in chronic or partial-chronic studies with marine organism. However, unless probabilities for chronic exposure to sewage sludge at dumpsites can be defined, it is debatable whether these complex tests should be conducted. At present, there is no accepted approach to use acute toxicity data in environmental situations for wastes such as sewage sludge, where waste concentrations and bioavailability of contaminants are expected to be quite variable over time. Hansen (in press) has pointed out that acute toxicity tests are most appropriately used to measure relative toxicity of substances or relative

sensitivity of species to a particular substance and not to predict environmentally acceptable concentrations.

Presently used mysid and Atlantic silverside acute bioassays, with some minor modifications, should provide a reliable and reproducible measure of acute toxicity of sewage sludge to characterize the toxicity of this waste. However, use of this acute toxicity data alone to estimate environmentally acceptable concentrations is questionable. Rather, environmental acceptability must be evaluated by more extensive investigations which estimate waste exposure concentrations in the field over time and contrast these against the potentials for adverse effects and bioaccumulation of contaminants as determined in appropriate tests with marine organisms. Such a hazard assessment strategy has been discussed by Gentile et al. (in press) and should form the basis for a scientifically credible procedure to evaluate the environmental safety of ocean dumping of sewage sludge.

5. CONCLUSIONS

1. Acute toxicity test methods developed at ERLN to test sewage sludge revealed that the three metropolitan sludges tested had generally low acute toxicities. The 96 h LC50 values observed ranged from 1 - 10% whole sludge for fish; 0.5 - 2.5% for mysids; and 0.03 - > 0.8% for copepods.

2. In most cases, similar results were obtained for fish and mysids using newly developed methods and tests presently used for regulatory decision making. This indicates that the present, easier to conduct tests may be satisfactory to measure acute toxicity of sludge relative to other wastes or to document changes in acute toxicity of sludges produced with time at a given treatment plant. One exception to this conclusion may be the fish bioassay for aerobically produced sludge. The newly developed larval fish test indicated lower acute toxicity for this type of sludge (sample #14) than the presently used method. Also, improvements in the experimental design of present tests are needed.

3. Experimental designs of the acute tests presently used to monitor toxicity of ocean barged sludge are deficient in that they does not consistently produce results suitable for statistical analysis. Therefore, relative toxicity of different samples from the same plants, samples from different plants and the sensitivity of test species cannot be validly compared. Minor increases in number of organisms per treatment, number of treatments and logarithmic dilutions between treatments used should remedy this deficiency.

4. The acute toxicity of sewage sludge from a given treatment plant may be quite variable over time, as shown by high coefficients of variation for seven within-plant comparisons of quarterly LC50 values.

5. Toxicity of sewage sludge was observed to increase up to an order of magnitude during storage at the Middlesex County Utilities Authority wastewater treatment plant.

6. Mysids (Mysidopsis bahia) are usually 1.4 to 3.5 times more acutely sensitive to sewage sludge than juvenile Atlantic silversides (Menidia menidia) based on 76 regulatory toxicity tests on sludge from seven metropolitan wastewater treatment plants. Our tests showed that copepods may be more acutely sensitive than this mysid by as much as a factor of 9.

6. RECOMMENDATIONS

1. Present regulatory bioassays and species tested are sufficiently sensitive to characterize acute toxicity of sewage sludge and with minor modification their use should be continued.

2. Modification in the experimental designs of presently used tests should be made so that LC50s and confidence intervals can be calculated by the probit method. Changes required include increasing number of waste concentrations to five or more, plus a control, and diluting waste treatments in a regular series (e.g., by a factor of 0.5 or 0.6, beginning with the highest concentration) spanning the probable range of toxicity for a particular treatment plant. An increase in the number of individuals tested will increase test precision.

3. Mysids (Mysidopsis bahia) and Atlantic silversides (Menidia menidia) remain species of choice for acute toxicity tests of sewage sludge for waste characterization purposes. They are used in marine testing because they are available through culture or capture, survival is acceptable in culture and testing, and they are reasonably sensitive.

4. Acute toxicity tests can be used to judge the relative toxicity of sludges, however, they should not be used to estimate environmental hazards of sewage sludges. Acute toxicity tests provide only the first evidence on potential effects of sludges, but this information must be contrasted with potential exposure information. Reasonable evaluations must consider sensitivities of dump site species contrasted with realistic exposures. Knowledge is not available to do this accurately, nor to evaluate secondary impacts of nutrient and organic material additions. This lack of a basic understanding of the potential for either direct or indirect biological effects in the context of exposure conditions at a

dump site supports the need for a more comprehensive research program. This research should follow a hazard assessment approach, such as described by Gentile et al. (in press) to assure that the research is conducted in the context of dump site conditions.

5. At treatment plants where sludge toxicity may vary between and within the storage tanks, the protocol for sampling barged sludge should specify at least two subsamples taken at the beginning and conclusion of pumping each storage tank. Present practice of compositing three subsamples taken at the beginning, mid-point and conclusion of barge filling may not give a composite representative of the two storage tanks pumped.

6. One outstanding research issue concerns the contrasting LC50 values produced by the present and revised fish test with aerobically produced sludge from the Middlesex County treatment plant. In the event the fish test is important for regulatory decision makers, additional tests should be conducted to verify observed differences, then to determine the reason for the differences, e.g. whether it is vigorous aeration which results in a higher LC50 or whether the Menidia beryllina larvae used in the revised test are less sensitive to sewage sludge than Menidia menidia juveniles.

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APPENDIX 1.

Sewage Sludge Samples Collected for Acute Toxicity Test Test Evaluation

Dates and manner of collecting sewage sludge samples at Middlesex County Utility Authority (NJ) (Samples #1-12, 14), Bergen County Utility Authority (NJ)(Sample #13) and Yonkers Joint Waste Water Treatment Plant, Westchester County (NY)(Sample #15). All samples were collected by ERLN personnel, unless indicated.

SAMPLE	COLLECTION DATE	METHOD AND POINT OF COLLECTION
#1	5/4/82	Bottom of a storage tank, via recirculation pump
2	9/22/82	Same
3	11/16/82	Same
4	12/21/82	Sludge line at base of gravity thickener, prior to storage
5	1/31/83	Same
6	3/22/83	Bottom of storage tank, via recirculation pump
7	5/4/83	Same
8	6/7/83	Sludge line at base of gravity thickener, prior to storage
9A	7/8/83	One-half of sample from bottom of #3 storage tank (filled to 22 ft.) and one-half from the tank currently being filled (#4, filled to 11 ft.)
9B	7/7-7/8 84	Composite sample collected by plant operators while pumping sludge to barge. One-third taken initially, one-third after barge one-half full, one-third when barge full.
10	9/10/84	Composite sample taken while pumping storage tanks to barge. One-third taken after one hour of pumping (storage tank c.a. one-third empty), one-third taken 15 min. after second tank pumping began, one-third taken 1 hr 15 min later from same tank.

SAMPLE	COLLECTION DATE	METHOD AND POINT OF COLLECTION
11	11/26/83	Composite sample of sludge from two storage tanks. One-half from tank #4 (80% of tank emptied on 8/24 - 8/25) and one-half from tank #1 (tank reported to be filled for at least 2 days).
12	2/3/84	Sludge line at base of gravity thickener, prior to storage.
13	3/5/84	Bergen County: Composite of barged sludge collected by plant operator. Samples taken every one-half hour during the c.a. 2 1/2 h of pumping to barge.
14	4/9/84	Middlesex County: Composite of barged sludge collected by plant operator per #9B procedure.
15	4/12/84	Yonkers Joint Treatment Plant, Westchester County: Composite of barged sludge collected by plant operator, one-third taken 20 min after starting sludge pumping to barge, one-third when barge is half full and one-third 30 min before barge is full.

APPENDIX 2.

Methods to Measure Dry Weight and Dissolved Oxygen Concentration of Sewage Sludge Bioassay Treatments

1. Protocol to Obtain Dry Weight Measurements of Sewage Sludge Bioassay Exposure Concentrations.

The sample must be collected from a well agitated container, keeping particles in suspension and preventing clumping, in order to assure a representative sample. Samples must be collected prior to adding animals since test organisms should not be exposed to such agitation.

Filtration and Weighing Procedure

Filter Paper Preparation

1. Place Gelman GF/AE 47 mm 0.3 μ m filter on apparatus with tweezers.
2. Turn on pump.
3. Rinse filter with 3 separate 20 ml rinses of deionized water. Use prefiltered deionized water (DI) (optional; see suggestions).
4. Turn off pump to release vacuum.
5. Remove filter with tweezers and place in clean aluminum planchette.
6. Dry at 103°C for 1 hour.

Sample Filtration, Drying and Weighing

1. Transfer filters to dessicator.
2. Weigh on a balance which is reliable to 0.0001 g.
3. Place filter on apparatus with tweezers. Turn on pump.
4. Wash with 1 - 50 ml rinse of DI.
5. Filter sample.
6. Wash sample with 3 - 10 ml rinses of DI.
7. Turn off pump to release vacuum.
8. Remove filter with tweezers and place in planchette.
9. Dry in oven at 103°C for 3 - 4 hours.
10. Transfer filter to dessicator.
11. Weigh.

Suggestions

1. Select number of filter papers needed plus several extras.
 2. Filter pre-washing is optional since filter weight loss occurs with washing. Weight loss continues with additional washings with no simple trend apparent, precluding use of a correction factor. The weight loss is small (0.002 - 0.003%), however, should 0.2 to 0.3 mg be a large % of the weight of a filtered material, it is advisable to use a filter other than the Gelman glass filter.
 3. If filter paper is to be rinsed, estimate amount of distilled water needed plus 200-300 ml extra. Filter DI for rinsing through a 0.3 μ m Gelman GF/AE filter and reserve. Before collection of the filtered DI into the filter reservoir and a storage reservoir, wash both several times with the filtered DI.
 4. If an undiluted sewage sludge sample counts $\approx 38,000$ on the Coulter Counter or is ≈ 0.6 ppm, a 100 ml sample should be adequate to obtain an accurate dry weight.
 5. Determine the size of the sample needed for the lowest bioassay concentration. For each doubling in concentration, the sample size can be reduced in half.
 6. If the sample collected for the lowest concentration blocks a filter for more than 5 minutes, discard the sample and filter and try a smaller sample size.
 7. The dry weight of any sample should weigh more than 1.00 mg if balance is precise only to 4 decimal places. Any weight less than 1.00 mg will yield only one significant digit.
 8. Turn off the filter pump before removing a wet glass fiber filter. This reduces the chance of leaving glass fibers on the apparatus which could increase the variability in initial and final weights.
-
2. Protocol for Taking Dissolved Oxygen Measurements, Using YSI Oxygen Meter, with Stirring Probe.

Procedure

1. Make sure there are no air bubbles under the probe membrane. If so, change membrane.
2. Take a sample of clean seawater at the salinity and the temperature of the bioassay solutions. Saturate this water with air using an aerater for 15 minutes. Read the temperature, using the thermometer. This is the calibration medium.
3. Make sure the oxygen probe is inserted in proper jack in the meter.

4. Switch instrument to RED LINE position and adjust meter to RED LINE with control on the front panel.
5. Place probe in calibration medium. Stirrer adapter MUST be plugged in and switch on when taking readings.
6. Switch to ZERO position and adjust meter to zero with ZERO control.
7. Switch to 0-10 or 0-20 ppm position and adjust meter to calibration value with CAL control. Calibration value should be read off chart for the proper temperature.
8. Place probe in sample of bioassay solution and allow needle to steady before reading value.
9. Turn meter and stirrer off between readings.
10. The oxygen level may be read directly from the meter in mg/l.

Cleanup

1. Rinse probe with copious amounts of deionized water.
2. Replace probe in a flask containing clean deionized water.

APPENDIX 3

Environmental Research Laboratory, Narragansett, Bioassay Procedures

Fish

Larval fish bioassay chambers consisted of 1.2 liter crystallizing dishes (20 cm diameter X 8 cm deep) held in a temperature controlled water bath. Each dish was aerated by a single Pasteur pipette connected to an air line. A total of six or seven treatments, including controls, were used in all tests, with two replicates per treatment and twelve fish per replicate. Control and sewage sludge treatments were prepared the morning of a test, added to the exposure dishes, and vigorously aerated for at least one hour prior to the addition of test organisms. Salinity and oxygen (see Appendix 2 for method) were measured in each dish just prior to adding the organisms.

Two to four week old Menidia spp. larvae were obtained for testing by gently capturing and transferring them from the culture aquaria with a 60 ml beaker. Larvae were placed in thirteen 150 ml round glass culture dishes, added in random allotments of two to three fish until each dish contained a total of 12 larvae. In assays on sludge sample #8, 11 and 13, fish were left in these dishes for approximately 1 h. Any fish which appeared injured or showed abnormal swimming behavior were removed and replaced with randomly selected larvae. Problems with control mortality with M. beryllina during the test on sample #13 bioassay called for changes in the pre-exposure holding procedure. For the assay on sample #14, fish were held in 150 ml dishes 20 h prior to testing and all dead and abnormally-appearing individuals replaced. Following this holding period, fish were released in each exposure chamber by gently sinking

and removing a randomly selected holding dish in the test chamber. Finally, for the assay on sample #15, fish were placed directly into the exposure chambers in 1 liter of filtered seawater and held for 24 h and dead and abnormal larvae were replaced. This latter procedure is recommended for tests using fish larvae. This test was started by adding a previously aerated mixture of sludge and filtered seawater to each exposure chamber to achieve the desired sludge concentration and volume. One to two day old Cyprinodon variegatus were handled and tested as described for Menidia spp., except initial collection from the culture aquaria was by a large bore pipette, and pretest holding to limit control mortalities was unnecessary.

During assays, live and dead larvae were enumerated daily and water temperature, salinity, and dissolved oxygen measured in each exposure chamber. Larval fish of both species were held under 100 LX cool white® fluorescent illumination with a 12L:12D photoperiod and fed newly hatched brine shrimp at approximately 60% their body weight per day. Test solutions were held at $22 \pm 2^{\circ}\text{C}$ and vigorously aerated throughout the test to maintain high oxygen concentrations ($> 85\%$ for samples #13 to 15); salinities ranged from 27-35 ‰. Even with vigorous aeration, the particulate phase of both aerobic and anaerobic sludges immediately began to fall out of suspension and flocculate upon contact with seawater. At the termination of the 96 h assay, the lower concentration test solutions were almost clear, but higher concentrations remained highly turbid. Therefore, to enumerate larvae, test solutions were passed through a seive and fish placed in clean water for enumeration. Missing fish were assumed to be dead. Tests in which the control mortality exceeded 10% were repeated.

Mysids

Acute 96 h static and static-renewal mysid lethality tests were conducted in 1.2 liter crystallizing dishes (190 x 100 mm) with stir bars and an air lift system designed to keep the sludge in suspension and maintain a consistent exposure concentration (Fig. A2-1). Each crystallizing dish contained three replicate exposure cups containing test organisms. Glass racks held cups above the bottom of the dish. Exposure cups were constructed from eight cm diameter wide mouth glass jars with bottoms removed and covered by 250 mm nylon mesh.

Acute 96 h lethality tests were initiated with five, randomly distributed 24 h old post-larval mysids in each cup in the five toxicant concentrations and the control. Position of each dish was also randomized. Each exposure cup was fed approximately 700 24 h post-hatch A. salina daily. Survival was monitored daily by removing exposure cups and placing them into a crystallizing dish containing clean seawater and a glass rack. The cups were gently shaken in the seawater to remove particulates and enhance visibility of test organisms when illuminated from beneath. Survivors were counted, dead mysids removed, and the exposure cups replaced in the exposure dishes.

Mysids bioassays were maintained at $25 \pm 1^{\circ}\text{C}$, 30 ‰ salinity, and 100 lx cool white® fluorescent illumination on a 14L:10D cycle. Temperature was monitored by a continuous temperature recorder; dissolved oxygen and salinity were measured daily.

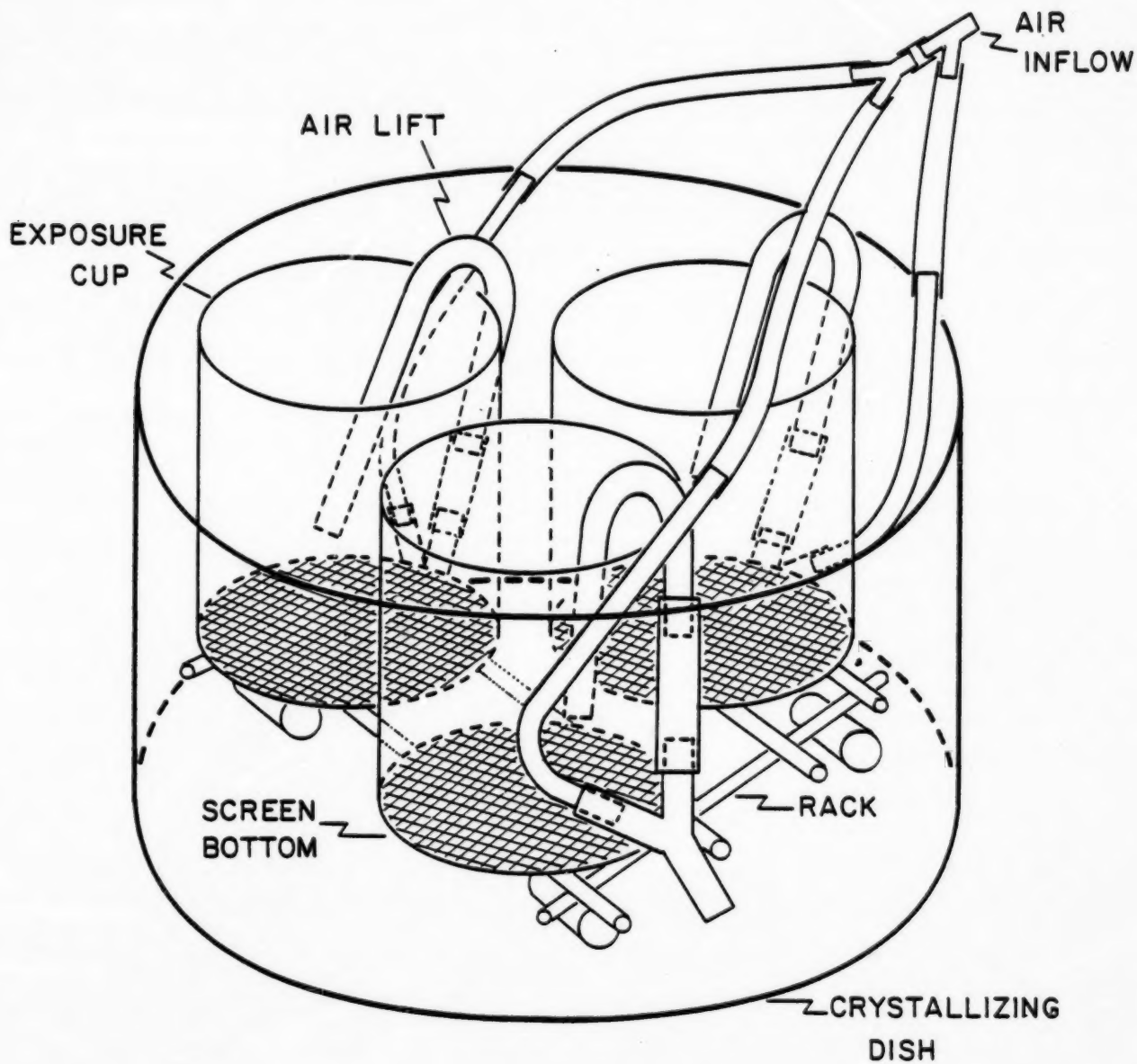


Figure A3-1. Exposure chamber for mysid test.

Copepods

All copepod toxicity tests were 96 h static tests with 24 h renewals. Each treatment was composed of four 100 ml replicates containing five females; 20 animals per treatment. Treatments consisted of six sludge concentrations plus a control. Sludge concentrations generally were below 1% since copepods are extremely difficult to locate and observe in higher turbidities. Treatment dilutions were such that partial kills required for probit analysis (ASTM, 1980) were usually obtained. Dilutions of sludge were mixed from highest to lowest concentration by diluting each higher concentration by a factor of 0.5, e.g., 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125%. Each treatment was vigorously aerated for a minimum of one hour at 12°C before adding copepods.

Tests were begun using newly matured female E. herdmani. Approximately 200 females were removed from the stock cultures within five days prior to testing and fed daily. For an assay, individuals were placed sequentially into 50 ml beakers (one for each replicate) containing 10 ml of seawater until all beakers contained five females. Care was taken throughout this process to maintain water at the culture temperature ($15 \pm 2^\circ\text{C}$). All beakers were placed at the experimental temperature (12°C) for a minimum of 15 min before introduction into exposure flasks. The order of the beakers was rearranged to insure a random assignment to each experimental replicate. The procedure for bioassays with P. minutus was comparable.

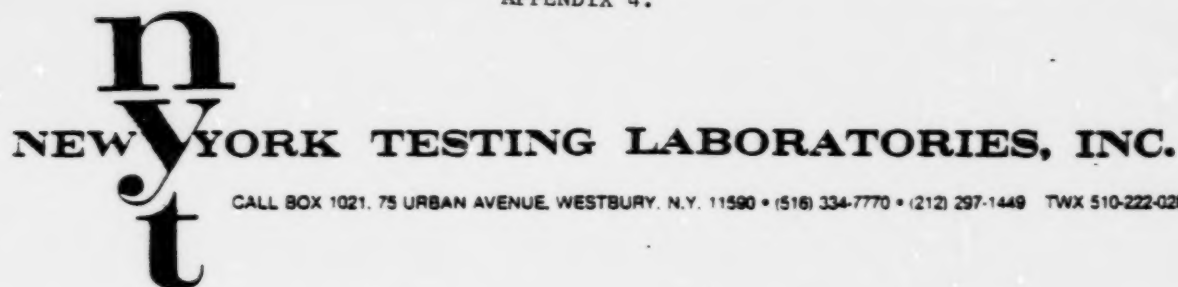
Copepod toxicity tests were conducted in 250 ml Erlenmeyer flasks containing 100 ml of test solution. Flasks were placed on orbital shakers within an incubator at $12 \pm 2^\circ\text{C}$. The rotational motion kept sludge particles

in suspension and enhanced gas exchange, maintaining the dissolved oxygen in the test solutions $\geq$ 60% saturation. Every 24 h, test solutions were gently poured from the Erlenmeyer flasks into an 11.4 cm Carolina culture dish. Flasks were gently washed with dilution water of the same temperature to free any animals stuck on the sides. Dead copepods were recorded and removed. Missing animals were noted and sample sizes were reduced accordingly during the data analysis. Living copepods were gently transferred to appropriate flasks of freshly mixed test solutions using a $\geq$ 2 mm i.d. glass pipette. Animals were always released under the water's surface to avoid damage incurred by dropping the animals through the surface tension. Copepods were not fed phytoplankton during tests.

It was sometimes difficult to determine if copepods were dead. Therefore, judgement criteria were required. With some sludges, a temporary anesthetic effect was sometimes observed. Frequently, animals that did not respond to prodding (a generally accepted criterion for death (ASTM, 1980) were observed actively swimming the following day. Therefore, the criteria for death were animals that: (1) were decaying; (2) were totally opaque, either white, pink, or orange; (3) had limp appendages that swayed freely in light water movement; and (4) showed no gut movement for 60 sec in the 2nd to 4th metasome when observed from the dorsal side.

The above procedures are recommended for acute tests with copepods. Initial tests on sludge samples #6 through 11 deviated from these procedures in three aspects, which are described in the attached report.

APPENDIX 4.



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MANUAL OF PRACTICE

BIOASSAYS

The following manual is to be used as an aid for technicians and laboratory management engaged in the performance of aquatic bioassays. The manual is not a duplication of regulatory procedure, but a clarification of the procedures. The manual puts forth the specific way the regulatory bioassays procedures will be performed at our facility. The following documents are considered in this manual.

1. Ecological Evaluation of Proposed Discharge of Dredged Material into Ocean Waters 7/77 U.S.E.P.A./ U.S.C.O.E.
2. Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms EPA-600/4-78-012.
3. Bioassay Procedures for the Ocean Disposal Permit Program EPA-600/9-78-010.
4. Standard Methods for the Examination of Water and Wastewater 15th Edition.
5. State of New Jersey Subchapter 6, 7:18 Criteria and Procedures for Bioassay Testing and Analysis.
6. N.Y.C.O.E. Guidance for performing tests on dredged material to be disposed of in Ocean Waters.
7. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians EPA-660/3-75-009.

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I. Test Performance

1. Dredged Material Bioassays

Sample preparation follows (reference 1). A 4:1 sea-water: sediment mixture is made and mechanically stirred for ½ hour. The supernatant is pulled off (siphoned) after 1 hour setting, and restirred before splitting or separation for filtration. This ensures equal amounts of particulate matter in each container. Containers are split into two groups one is labeled "to be filtered" and the other "suspended particulate phase". This eliminates inadvertent concentration of sample by half filtering a container which is to be used for suspended particulate bioassays.

A. Liquid Phase

1. Skeletonema Costatum

Tests are performed according to references (6) and (1)

A minimum of 4 cell counts are performed at each test interval on all test flasks. On the final day of the test, 6 cell counts are performed.

The form at appendix 1 is used for the results. This form is entitled Phytoplankton Bioassay Bench Sheet (Appendix 1).

2. Menidia Menidia

This test is performed according to references 6 and 1.

Reporting forms used are as follows:

- a) Mortality Analysis (Appendix 2)
- b) Bioassay Bench Sheet (Appendix 3)
- c) Menidia Menidia sublethal effects (Appendix 4)

Survival results and sublethal effects are observed at 0, 4, 8, 24, 48, 72 and 96 hours.

Environmental parameters are recorded for 0, 24, 48, 72 and 96 hours.

I. Test Performance - cont'd

3. Mysidopsis Bahia

This test is performed according to reference 6 and 1.

Reporting forms are as above Appendix 2 & 3; and Appendix 5 (Mysidopsis Bahia sublethal effects). Readings are taken as above

B. Suspended Particulate Phase

1. Acartia Tonsa

The test is performed according to references 6 and 1.

Reporting forms are Appendixs 2 & 3 and Appendix 6 (Acartia tonsa sublethal effects).

Readings are taken as above.

2. Menidia Menidia

This test is performed according to references 6 and 1.

Reporting forms are the same as Menidia Menidia liquid phase forms.

3. Mysidopsis bahia

This test is performed according to references 6 and 1.

Reporting forms are the same as Mysidopsis bahia liquid phase forms.

All tests are performed with aeration. Mysids are fed during the test.

C. Solid Phase

1. Nereis Virens

This test is performed according to references 6 and 1.

The Nereis are tested in separate aquaria from the other solid phase species. All aquaria are supplied with flow through lines (intake & outlet) and an aeration line. Survivals are checked daily and recorded on solid phase bioassay survival sheets (Appendix 7). Environmental parameters are checked daily and recorded on Environmental Parameter Sheet (Appendix 8).

I. Test Performance - cont'dC. Solid Phase - cont'd

Sublethal effects are observed daily and recorded on *Nereis virens* sublethal effects (Appendix 9).

2. Mercenaria Mercenaria3. Palaemonetes Sp.

These tests are performed according to references 6 and 1.

These species (clams and grass shrimp) are run in combined tanks, since there is little or no species interactions. Reporting forms are shown in Appendix 7 & 8, mentioned above, and 10 (*Mercenaria mercenaria* sublethal effects) and 11 (*Palaemonetes* sublethal effects).

Solid phase animals prior to testing are analyzed for the presence of bioaccumulation substances required. (PCB's, petroleum hydrocarbons, Hg, Cd.). If the specimens are clean, the batch is used for testing. After the solid phase bioassay period (ten days), the test organisms are removed from the sediment and depurated in flowing seawater for two days. At this time the animals are tissued, frozen, and delivered to our Westbury facility for testing.

D. Test Reporting

All test raw data is submitted as the report. In addition a final report sheet (Appendix 12) is supplied giving a summary of results and organism source and acclimation data. LC and EC are determined by graphical interpolation. The dredged material grain size and geologic field data is also submitted with the report.

Chemical testing is provided on the elutriate (liquid phase with site water), site water, and bulk sediment including separate stratification layers.

2. Sludge and Industrial Wastes BioassaysA. Skeletonema Castatum

The test is performed according to reference 3 section 8. Static method for acute toxicity tests with phytoplankton.

I. Test Performance - cont'dA. Skeletonema Castatum - cont'd

Wet settled sludge is used to produce the tested extract. Volumes are made in equivalents of these in the procedure, but in lesser amounts, since this is all that is required for testing. The initial inoculum is 8.0×10^3 / ml - 1.0×10^4 / ml. The tests are monitored daily. At 96 hours the flasks (triplicate) are counted with six hemacytometer counts per flask. The results are recorded on the form in Appendix 1. The data form is the final report.

Note: Industrial wastes are pH adjusted prior to testing (as required by the Federal Register). The adjustment dilution is taken into account when determining test concentrations.

B. Menidia Menidia

This test is performed according to reference 3, section K.

Natural seawater is used for testing. The tests are performed both with and without aeration for the following reasons:

- 1) Aeration affects pH, and increases particulate and bacterial residence time in the water column.
- 2) Without aeration tests show low dissolved oxygen tensions which could occur in an environment with little water circulation.

The reporting form is shown in Appendix 3.

The aeration variations above have created LC_{50} variations between with and without aeration tests with two-tailed effects. It is therefore, NYTL's policy to perform both tests and report all data.

C. Mysidopsis Bahia

The test is performed according to reference 3, section F.

Natural seawater is used for test dilution water. Mysids are fed during the test period. The results are reported on the form shown in Appendix 3.

D. Test Reporting

Data sheets are reported. A final report sheet (Appendix 13) containing organism acclimation data, source of organisms, and a summary of results.

I. Test Performance - cont'd3. Industrial Effluents

These are tested in accordance with reference 2 and reference 5 when required.

4. Basic Tests

These are performed according to reference 7.

II. Quality Control Program1. Sample Collection and Handling

Sample collection is performed in accordance with project requirements. Samples are taken and identified by location, date and time taken, preservatives added (if any), and sampler. The sampler delivers the sample or transfers it to a driver. A chain of custody form is filled out and moves with the sample. The sample may be delivered to either the Westbury or Oceanside facility. The chain of custody form or its copy (for a split sample) accompanies its movement. In the laboratory the chain of custody form is signed by the person receiving the sample. During any transfer step, the sample receiver must be reasonably sure that the sample has been taken, held, and/or transported under proper conditions.

2. Test Organism Culture, Collection and HandlingA) Phytoplankton (Skeletonema Costatum)

1. Phytoplankton species are cultured in the laboratory.
2. The culture is maintained using microbiological aseptic technique.
3. The species are maintained with use of 7 day flash cultures in medias required for tests (or their prerequisites).
- * Transfer cultures must be observed microscopically before use in the propagation of the culture. The technician must discard any culture with contamination.
4. Test inoculums are taken from 7-14 day cultures in logarithmic phase growth. Stock or previous test controls may be used as test inoculums.
5. Defined media (synthetic seawater) will be used as dilution water.
6. All stock and test flasks will be dated on the flask.

B) Zooplankton (Acartia Tonsa)

1. Acartia tonsa may be collected, cultured or purchased.
2. Collected specimens will be identified microscopically. Several specimens will be observed from a collection. After microscopic observation; visual inspection will be used to separate species.
3. Due to the short life cycle (17 days) acclimation time will be 2-4 days with feeding of phytoplankton.
4. Cultured or purchased specimens will be used as young adults.

C) Mysidopsis Bahia

1. Mysidopsis bahia will be cultured or purchased.
2. Culture records will be kept on holding tanks including the following parameters;
 - a) temperature
 - b) salinity
 - c) dissolved oxygen
 - d) births
 - e) mortalities (include those used for tests in this category)
3. Purchased specimens will be verified as Mysidopsis bahia.
4. Tests will be run on test mysids from the same batch. Mysids will be randomly distributed to the test tanks.

D) Menidia Menidia

1. Menidia will be field collected, purchased or cultured.
2. Field collected specimens will be quarantined for a 14 day period (minimum) for acclimation and possible prophylaxis. Handling during this period will be recorded on the field collection and acclimation data sheet.
3. After acclimation, the fish will be assigned to a holding tank and assigned a batch number. Batches may contain fish from several different collections, but all will have a minimum of 14 days acclimation. Batches will show less than 10% mortality for 7 days prior to testing.

II. Quality Control Program - cont'd

D) Menidia Menidia - cont'd

4. Records will be kept of holding tank parameters including:
 - a) temperature
 - b) salinity
 - c) dissolved oxygen
 - d) mortalities (including cause of death)
 - e) ammonia + nitrites
 - f) water changes
 - g) feeding schedules + type
5. Fish in holding tanks or for specialized tests at conditions different from required test conditions will be reacquainted as required prior to testing.
6. Only actively feeding fish will be used in testing.
7. Menidia will not be fed during the test period.

E) Other Fish and Macroinvertebrates

1. These specimens will be handled as Menidia Menidia above.

3. Laboratory Equipment + Supplies

- A. Air supply is provided by oil-less compressors. Air filters are changed monthly.
- B. Testing systems including flow-through piping, tanks, temperature control units, valves, filters, glassware and fittings are composed of nontoxic substances.
- C. Temperature monitoring devices are sensitive to 0.5 deg.C. and are calibrated annually against a standard NBS certified thermometer.
- D. Salinometers are calibrated daily against a standard KCL solution. The results are recorded and maintained.
- E. Dissolved oxygen meters are calibrated daily by methods recommended by the manufacturer (operation manual). As a double check, dissolved oxygen results are also tested using oxygen saturated water. Also a periodic check, is performed using a Winkler method titration.

II. Quality Control Program - con'tLaboratory Equipment + Supplies - cont'd

- F. The autoclave is maintained in good operating condition with semi-annual servicing. Records are kept of:
- 1) Exact times of sterilization cycle including:
 - a) starting time
 - b) time to reach sterilization temperature (121 deg.C.)
 - c) Completion of sterilization cycle
 - d) time supplies are removed from the autoclave
 - 2) Supplies autoclaved.
 - 3) Internal temperature check with maximum thermometer.
 - 4) Sterilization check using a live bacterial culture is performed on a monthly basis.
- G. The walk-in refrigerator is of sufficient size to accept several samples and stocks of reference materials. The refrigerator is maintained at 1.0-4.4 deg.C. The temperature is recorded daily from liquid immersed calibrated thermometers. The thermometers are located at points near the condensing unit (to monitor low extremes) and at points furthest from the unit (to monitor for high extremes) samples in the refrigerator are held with spaces between each stack. Containers (generally 5 gallon) are not stacked more than 3 high.
- H. Lighting systems are checked daily for timing cycle. Fluorescent bulb quality is checked by light meter on a monthly basis. Bulbs are replaced if a variation occurs.
- I. Shaking systems are maintained annually using a control algal assay for all single flask holding locations. This mapping system can indicate variations in micro-environmental parameters for each test unit area. The assay results are computed statistically, and any unit areas not within 95% confidence limits are not used for definitive testing.

II. Quality Control Program - cont'd.

- J. Bioassay test areas (rooms or chambers) are monitored daily for temperature. The results are recorded and maintained.
- K. Holding tanks are maintained with records of pH., D.O., salinity, temperature, nitrites, ammonia, mortalities, and sublethal effects.
- L. Chemical stock solutions are kept for no longer than 1 year with concentration, date prepared and preparer's initials.
- M. Test dilution is analyzed weekly for the substances used in bioaccumulation testing (reference 6).
- N. Sterilization of the dilution water is checked monthly using standard plate count (bacteria/ml) and total coliform (MPN/100ml) analyses.
- O. Chemical analyses such as hardness and alkalinity are performed according to reference 4. The standards required by this reference are used as a quality control.
- P. Balances are maintained, and calibrated annually. S-standard weights are used for monthly calibration. The balance must detect a weight of 100mg accurately.
- Q. Laboratory grade water is tested semi-annually for suspended solids, T.O.C., unionized ammonia, residual chlorine, total organophosphorus pesticides, total organochlorine pesticides and PCBs. The maximum allowable detectable limits are as follows:
- | | |
|-----------------------------------|---------|
| Suspended solids | 20 mg/l |
| T.O.C. | 10 mg/l |
| Unionized ammonia | 20 ug/l |
| Residual chlorine | 50 ug/l |
| Total organophosphorus pesticides | 50 ng/l |
| Total organochlorine pesticides | 50 ng/l |
- R. Laboratory pure water will be analyzed monthly for pH, conductivity, and free chlorine, total alkalinity, and total hardness
- S. Laboratory pure water will be checked annually for trace metals, bactericidal propicities, and standard plate count.

II. Quality Control Program - cont'd.4. Standard Toxicant Testing Program

- A. A standard toxicant (SLS) is used as a bioassay standard.
- B. Standard toxicant testing is performed within 30 days of bioassay testing.
- C. Standards are performed on *Skeletonema Costatum*, *Menidia menidia*, *Mysidopsis bahia* and *Acartia tonsa*.
- D. Synthetic seawater is used for dilution water for standards to be reported with dredged material analyses. (Reference 6).

5. Laboratory Management

The bioassay laboratory supervisor is responsible for the establishment of the quality control program, and the performance of bioassays. Any procedural changes are made only by the bioassay supervisor. Procedural changes are described in this manual of practice.

The bioassay laboratory supervisor is required to report any changes to the analytical laboratory director.

APPENDIX 1Laboratory Grade Water Analysis

(Semi-annual)

1/25/83

Total Suspended Solids	< 1 mg/l
T.O.C.	2 mg/l
Unionized ammonia	< 0.04 ug/l
Chlorine residual	< 0.01 ug/l
Total organophosphorus pesticides	<10 ng/l
Total organochlorine pesticides + PCBs	<10 ng/l

Laboratory Pure Water

Total Alkalinity	< 5 ng/l
Total Hardness	< 1 ng/l
Specific Conductance	12uV/cm

APPENDIX 2.Water Suitability Test

(Bactericidal Properties Test)

12/20/82

Growth Inhibiting Substances

$$\text{Ratio} = \frac{\text{Flask B}}{\text{Flask A}} = \frac{160}{155} = 1.0$$

A ratio of 0.8 - 1.2 shows no toxic substances

Growth Promoting Substances

$$\text{Ratio} = \frac{\text{Flask C}}{\text{Flask A}} = \frac{140}{155} = 0.9$$

A ratio less than 1.2 shows no available source for bacterial growth.

Reference: Microbiological Methods for Monitoring the Environment

Water & Wastes EPA 8-78-017.

APPENDIX 5.

SUMMARY OF ACUTE TOXICITY DATA FOR SEWAGE SLUDGE REPORTED QUARTERLY BY THREE METROPOLITAN WASTEWATER TREATMENT PLANTS

Acute toxicity data were reviewed from quarterly sewage sludge assays from 1981 to the second quarter of 1984 for three sewage treatment plants: Bergen County, NJ, Middlesex County, NJ, and Yonkers Joint Waste Water Treatment Plant, Westchester County, NY. The purpose of the review was to evaluate test results of Atlantic silverside (Menidia menidia) and mysid (Mysidopsis bahia) assays from a contract laboratory and to determine the extent that they may be validly used for comparisons of sludge toxicity and species sensitivity. The assays were conducted by New York Testing Laboratories (NYTL). LC50 values and 95% confidence limits (if possible) were determined for each assay by four methods: 1) graphical interpolation (performed by NYTL), 2) binomial test, 3) moving average (Thompson, 1947), and 4) probit (Finney, 1971). The later three determinations were performed by the EPA Environmental Research Laboratory, Narragansett (ERLN), using computer software developed by Scephan (EPA, ERL, Duluth) and modified to include Abbott's correction for control mortality.

These data were analyzed in the following ways:

- 1) The percent success of applying each of four methods to estimate the LC50 for the test data was determined. Percent success was computed as the number of times a particular method could be successfully applied to the test data, for a given treatment plant, divided by the total number of tests performed during

the 1981-1984 time period, expressed as a percent. The criteria for "successful" application of a method are described in Stephan (1982). This is one measure of the adequacy of the experimental design. Ideally, an LC50 value and confidence limits should be obtainable for 100% of the tests reported.

- 2) Overall means and standard deviations (SAS Institute, Inc., 1982) for each of the three treatment plants and two test species were computed. These values may be used to indicate variability in response over the 2 1/2 year time period i.e., between test variability.
- 3) Ninety-five percent confidence intervals were computed, where possible, for each species, treatment plant, and method of LC50 estimation as a measure of within-test variability. The overall mean magnitude of the 95% confidence intervals was also calculated for the 1981-1984 time period for each treatment plant.

NYTL Experimental Design

Each quarterly 96h acute mysid or Atlantic silverside sewage sludge toxicity test performed by NYTL utilized either three, four, or five sludge concentrations plus a control. If an LC50 could not be determined by graphical interpolation upon completion of a 96h test, and sufficient sludge sample was available, a second test consisting of one sludge concentration plus a control was conducted. (Results of these latter tests were deleted from this data analysis since this procedure is not amenable to statistical evaluation.) Most tests employed static exposures, although in 1981 a few tests used the flow-through mode. Basic procedures

used in these tests are outlined in the New York Testing Laboratories, Inc. Manual of Practice (unpublished, Appendix 4). The mysid and Atlantic silverside tests for sludge were largely designed from the U.S. EPA (1978) Bioassay Procedures for the Ocean Disposal Permit Program.

Juvenile silversides were divided into two groups of ten fish per sludge concentration or control. Tests with one group were conducted without aeration; tests with the other group were aerated throughout the 96h test period. Fish were not divided between replicate containers for either of these tests.

Mysid assays consisted of two groups of ten juvenile animals per sludge concentration or control ($n = 20$ mysids per treatment). Aeration was provided to all test containers throughout the 96h test.

LC50 values were determined by NYTL using graphical interpolation (Rand et al., 1975). Ninety-five percent confidence limits are not obtainable by this method.

Results

The percent of success values (Table A5-1) indicate that the NYTL experimental design is inadequate to consistently obtain LC50 values and confidence intervals by any method for LC50 estimation. The results obtained from the M. menidia tests performed without aeration were the least satisfactory, with only 57% of these tests yielding an LC50 by graphical interpolation, which is the least rigorous method. LC50 values were most consistently obtainable for the mysid test data, however, even the mysid tests did not uniformly yield LC50 values by any method other than graphical interpolation.

The moving average and probit methods could not be used on over half of the NYTL fish test results. Furthermore, these methods occasionally yielded erroneously large 95% confidence intervals, e.g. a value of 72.31% ($= 2 \times 36.16$) was estimated by the probit method for one mysid test with Bergen County sludge (Table A5-2). The binomial method could be used frequently, but the magnitudes of the 95% confidence intervals obtained were large relative to the mean LC50 values, indicating large within-test variability (Table A5-2). This again points to the inappropriateness of the LC50 determination methods for these data and the inadequacy of the chosen experimental designs to provide sufficient data for application of the more rigorous methods of LC50 estimation (e.g., probit analysis).

Means and standard deviations for all LC50 data by treatment plant and species are reported in Table A5-2. Of interest for this review are the standard deviations, which are large, frequently 30-50% of the mean, indicating appreciable between-test variability over time in the LC50 values reported for these three treatment plants. Means of LC50 values by plant range from approximately 0.4 to 1% whole sludge for the mysid test, 1 to 2% for the fish test with aeration and 0.7 to 3% for fish without aeration. When interpreting these values, it should be remembered that each LC50 value reflects all responses of the organisms to test conditions, including variations in test water quality, animal condition, other seasonal effects, etc., as well as sewage sludge chemical contaminants. Hence variation between LC50 values may not always reflect differences in waste toxicity.

Discussion

The information required of an acute toxicity test will vary depending on the purpose of the test. For example, should only an order of magnitude estimate of acute toxicity be required, such as for screening purposes, an experimental design which produces high precision results may not be necessary. In this case, it also may be quite satisfactory to use a simple method to estimate the LC50, e.g., graphical interpolation. However, if test results are to be compared statistically, the experimental design should produce sufficient data to estimate the LC50 by the probit method preferably, or at the minimum, by the moving average or binomial method. Pairs of LC50 values estimated by the latter two methods can be evaluated for significant differences by comparing 95% confidence intervals (C.I.). If the C.I. do not overlap, the LC50 values are different ($P \leq 0.05$); if they overlap, the LC50's may or may not be significantly different. In contrast, if the probit method is used to estimate the LC50, a more rigorous procedure (Finney, 1971) is available to test for significant differences between pairs of values.

This review of quarterly sludge bioassays for three treatment plants has shown that the data produced were often insufficient to estimate reasonable 95% confidence limits. This was most frequently due to insufficient test concentrations surrounding the LC50 and, as a result, the partial kills of the test organisms required to calculate LC50s were not obtained.

Although replication of an acute test is not essential for LC50 determination (Stephan, 1977), replication within and between tests is

required for other statistical procedures, (e.g., analysis of variance (ANOVA), which might be used to determine a no effect level). Present test procedures for sewage sludge do not use replicate containers in the Menidia tests and only duplicate containers per treatment for the mysids. Also, no replicate tests are performed, precluding statistical assessment of within-laboratory variability vs. variability due to differences in sludge sample toxicity.

In conclusion, we recommend that improvements be implemented in experimental design of acute tests used to measure sewage sludge toxicity. An increase in number of treatment concentrations spaced at intervals of 0.5 to 0.6 and an increase in number of individuals tested per concentration should reduce test variance and produce sufficient data to apply the probit, preferably, or binomial or moving average models to estimate the LC50 with confidence limits. This is crucial in order to make valid comparisons between the toxicity of different sludge samples from the same plant or samples from different plants. Replication within and between tests should also be undertaken to quantify the sources of observed variability.

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Table A5-1. Percent of successful application of four methods to estimate LC50 to the New York Testing Laboratories acute toxicity data set (1981-1984). A total of 111 tests were conducted on sludges from three treatment plants; 37 tests each with Menidia menidia with aeration, M. menidia without aeration and with Mysidopsis bahia.

LC50 Determination Method				
<u>Species</u>	<u>Graphical</u>	<u>Binomial</u>	<u>Moving Average</u>	<u>Probit</u>
<u>M. menidia</u> ¹	81	76	43	43
<u>M. menidia</u> ²	57	54	24	30
<u>M. bahia</u>	97	86	70	84
Overall Mean	78	72	46	52

¹ tests performed with aeration

² test performed without aeration

Table A5-2. Mean LC50 values (% whole sludge) $\pm$ standard deviation for the New York Testing Laboratories 1981-1984 data for three wastewater treatment plants. Number of tests are in parentheses and $\pm$ one-half the mean magnitudes of the LC50 95% confidence intervals are in brackets. Note that confidence intervals could not be estimated for all LC50 values. Similarly, LC50s could not be estimated for all bioassays by binomial, moving average and probit methods since the design requirements for the methods could not always be met.

Treatment Plant	Species	Graphical	Binomial	Moving Average	Probit
Bergen Co., NJ	<u>M. menidia</u> ¹	2.31 $\pm$ 1.26(11)	2.34 $\pm$ 1.27(11) [$\pm$ 1.04](10)	1.63 $\pm$.31(5) [$\pm$.51](5)	2.10 $\pm$ 1.45(6) [$\pm$ 3.95](6)
	<u>M. menidia</u> ²	3.23 $\pm$ 1.19 (6)	3.31 $\pm$ 1.14(6) [$\pm$ 1.44](4)	2.69 (1) [$\pm$.64](1)	2.67 (1) [$\pm$.68](1)
	<u>M. bahia</u>	.71 $\pm$.19(13)	.72 $\pm$.22(12) [$\pm$.82](7)	.74 $\pm$.22(9) [$\pm$.28](5)	.79 $\pm$.22(11) [$\pm$ 36.16](10)
Middlesex Co., NJ	<u>M. menidia</u> ¹	1.30 $\pm$.39(7)	1.30 $\pm$.38(7) [$\pm$ 1.16](7)	1.19 $\pm$.39(5) [$\pm$.44](5)	1.11 $\pm$.33(5) [$\pm$.43](5)
	<u>M. menidia</u> ²	.73 $\pm$.37(8)	.73 $\pm$.36(8) [$\pm$.80](7)	.76 $\pm$.49(5) [$\pm$.28](4)	.78 $\pm$.44(6) [$\pm$.19](4)
	<u>M. bahia</u>	.43 $\pm$.21(9)	.43 $\pm$.23(8) [$\pm$.51](5)	.44 $\pm$.24(7) [$\pm$.15](5)	.49 $\pm$.26(8) [$\pm$.19](7)
Westchester Co., NY	<u>M. menidia</u> ¹	2.21 $\pm$ 1.05(12)	2.21 $\pm$.83(10) [$\pm$ 1.50](6)	1.68 $\pm$.70(6) [$\pm$ 6.15](4)	1.79 $\pm$.74(5) [$\pm$ 2.83](5)
	<u>M. menidia</u> ²	2.39 $\pm$ 1.42(7)	2.64 $\pm$ 1.40(6) [$\pm$ 1.15](6)	2.05 $\pm$ 1.62(3) [$\pm$.50](3)	1.84 $\pm$ 1.38(4) [$\pm$.64](3)
	<u>M. bahia</u>	.96 $\pm$.40(14)	1.01 $\pm$.50(12) [$\pm$ 1.00](8)	.95 $\pm$.54(11) [$\pm$.39](9)	1.12 $\pm$.61(12) [$\pm$.26](9)

¹ tests performed with aeration
² tests performed without aeration